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Mitotic factors from mammalian cells induce germinal vesicle breakdown and chromosome condensation in amphibian oocytes

(mitotic proteins/meiotic maturation/HeLa cell cycle)

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ABSTRACT **Cytoplasmic extracts of HeLa cells synchronized in various phases of the cell cycle were injected into fully grown *Xenopus laevis* oocytes to monitor the presence of factors that can induce meiotic maturation: i.e., germinal vesicle breakdown and chromosome condensation. Extracts from G1 and S phase cells had no activity. The maturation-inducing activity, which was found to be low during early and mid G2 phases, increased rapidly during late G2 and reached a peak in mitosis. The results of this study suggest that the factors that regulate the breakdown of nuclear membrane and chromosome condensation during mitosis, meiosis, and premature chromosome condensation appear to be very similar, if not identical, throughout the animal kingdom.**

The fusion between a mitotic and an interphase cell usually leads to the breakdown of the interphase nucleus and the condensation of chromatin into discrete chromosomes under the influence of the factors present in the mitotic cell. This phenomenon, which resembles the initiation of mitosis, has been termed premature chromosome condensation (PCC) (1). Mitotic cells of human origin (HeLa), upon fusion, can induce PCC in cells from a variety of animal species including mammals, birds, amphibians, fishes, and insects, and mitotic cells from these species can induce PCC in HeLa cells (2). The mitotic factors have been found to migrate to the interphase nucleus and subsequently become associated with the prematurely condensed chromosomes (3). In order to isolate and characterize the mitotic factors, it is essential to have an *in vitro* system in which PCC can be induced without resorting to cell fusion. Until now, however, we were unable to induce PCC in either whole cells or isolated nuclei by incubating them with mitotic extracts under a variety of conditions.

In our search for a suitable model, we found the amphibian oocytes desirable. In amphibians, meiotic maturation of ovarian oocytes involves breakdown of the nuclear envelope (germinal vesicle), chromosome condensation, and progression through the first meiotic division. This maturation process can be induced by incubating fully grown oocytes with progesterone *in vitro* (4–6). Meiotic maturation can also be induced in amphibian oocytes by injecting them with cytoplasmic extracts from maturing oocytes (7–9). Recent reports indicate that maturation-promoting activity is present not only in maturing oocytes but also in early cleavage stages of amphibian embryos, which undergo cell division with a high degree of synchrony (10). The activity of cytoplasmic extracts appears to fluctuate during the division cycle of the embryonic nuclei and reaches a peak during mitosis (10). Further, it was shown that introduction of somatic cell nuclei into maturing oocytes leads to condensation of chromosomes of the interphase nucleus (11). Because meiotic maturation appears to be similar to the induction of PCC, particularly with regard to breakdown of the nuclear membrane and condensation of chromosomes, we decided to test whether mitotic factors from mammalian cells could induce maturation in amphibian oocytes. If they could, we would be able to use this system as a biological assay for detecting the presence of the mitotic factors during the HeLa cell cycle. An abstract of this study has appeared elsewhere (12).

MATERIALS AND METHODS

Cells and Cell Synchrony. HeLa cells were grown as monolayer cultures at 37°C in Eagle's minimal essential medium supplemented with nonessential amino acids, heat-inactivated fetal calf serum (10%), sodium pyruvate, glutamine, and penicillin-streptomycin mixture (13). These cells have a cell cycle time of 22 hr consisting of 10.5 hr of pre-DNA-synthetic (G1) period, 7.0 hr of DNA-synthetic (S) period, 3.5 hr of post-DNA-synthetic (G2) period, and 1.0 hr of mitosis (14). Different methods were used to synchronize cells in various phases of the cell cycle. Briefly, a random population of HeLa cells was incubated with thymidine (2.5 mM) for 16 hr. Then the drug was removed by washing and cells were reincubated in regular medium for 8 hr, at the end of which thymidine was again added to the medium. After a period of 16 hr, the second thymidine block was released to obtain a synchronous population of S-phase cells. Synchronized S-phase populations having a labeling index of 95% were obtained by harvesting cells at 1 hr after reversal of the second thymidine block (14). In order to obtain pure populations of G2-phase cells, Colcemid (0.05 μg/ml) was added to the dishes 4 hr after the reversal of the second thymidine block and incubation was continued. At 7, 8, and 9 hr after reversal of the second thymidine block, Colcemid-arrested mitotic cells were removed by selective detachment and the cells that remained firmly attached to the dish were harvested by trypsinization to yield early, mid-, and late G2 populations. Because the cells were progressing through S phase into G2 in a synchronous fashion it is assumed that a majority of cells were in early, mid-, and late G2 periods at 7, 8, and 9 hr, respectively, after the reversal of the second thymidine block. This was further confirmed by a gradual decrease in labeling indices. For example, incubation of early, mid-, and late G2 cells with [^{3}H]thymidine [1.0 μCi/ml; specific activity 6.7 Ci/mmol (1 Ci = 3.7×10^{10} becquerels)] for 30 min and subsequent autoradiography of the fixed cells revealed labeling indices of 16%, 12%, and 2%, respectively. The mitotic indices

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Abbreviations: PCC, premature chromosome condensation; GVBD, germinal vesicle breakdown.

in all the three populations were less than 3%. To obtain mitotic populations, HeLa cells were first partially synchronized by a single thymidine block of about 20 hr duration. Four hours after reversal of the thymidine block, cells were incubated for about 10 hr in a chamber filled with N_2O at a pressure of 80 pounds/inch^2 (5.36 atm, 550 kPa). The rounded and loosely attached mitotic cells were selectively detached by gentle pipetting, which yielded a population with a mitotic index of 98% (15). In HeLa cells, mitotic block by N_2O is completely reversible. Incubation of N_2O-blocked mitotic cells under regular culture conditions for 3 hr yielded highly synchronous populations of G1 cells. Early and late G1 phase cells were obtained by trypsinizing cells at 3 and 7 hr, respectively, after the reversal of the N_2O block. Cells arrested in G2 phase were obtained by treating synchronized S-phase cells with *cis*-4-[{[(2-chloroethyl)nitrosoamino]carbonyl}amino]cyclohexane carboxylic acid (NSC-153174 or *cis*-acid) (75 μg/ml) for 1 hr immediately after the reversal of second thymidine block and then incubating for 24 hr in regular medium (16). The labeling and mitotic indices of these cells were 10% and 5%, respectively.

Preparation of HeLa Cell Extracts. Cytoplasmic extracts of cells from different phases of the cell cycle were prepared by suspending cells at a concentration of 20×10^6 cells per ml of extraction medium containing 0.2 M NaCl, 0.25 M sucrose, 0.01 M $MgSO_4$, 0.002 M ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA), and 0.01 M Na_2HPO_4/NaH_2PO_4, pH 6.5 (9). Cell extracts were obtained by hand homogenization in a Teflon pestle/glass homogenizer (20 strokes) at 4°C. The homogenate was centrifuged at 30,000 $\times g$ for 15 min in a refrigerated Sorvall RC5 centrifuge at 4°C. The supernatants thus obtained were used for the assay of maturation-promoting activity in *Xenopus laevis* oocytes. Protein concentration in the extracts was determined by the method of Bradford (17) with bovine serum albumin as the standard.

Preparation of *Xenopus* Oocytes. Oocytes were obtained by surgically removing a portion of the ovaries from *Xenopus* females. With a small incision that can be closed by a few stitches, multiple harvests of oocytes can be obtained from the same animal. All operations on oocytes were conducted using amphibian Ringer's solution supplemented with $MgCl_2 \cdot 6H_2O$ (0.12 g/liter). Oocytes were manually dissected from their follicles after pretreatment of the ovarian fragments with collagenase (1 mg/ml) (6).

Assay for Maturation-Promoting Activity. Cell extracts were assayed by injecting 65 nl into each oocyte. Injected oocytes were inspected for germinal vesicle breakdown (GVBD) after 2–6 hr. GVBD is detected by a depigmentation of an area of the animal hemisphere (16). Presence or absence of the germinal vesicle was also determined by dissection of the oocyte. Some oocytes were fixed in Smith's fluid, dehydrated with amyl acetate (18), embedded in paraffin, sectioned at 7 μm, and stained with Feulgen and fast green (19).

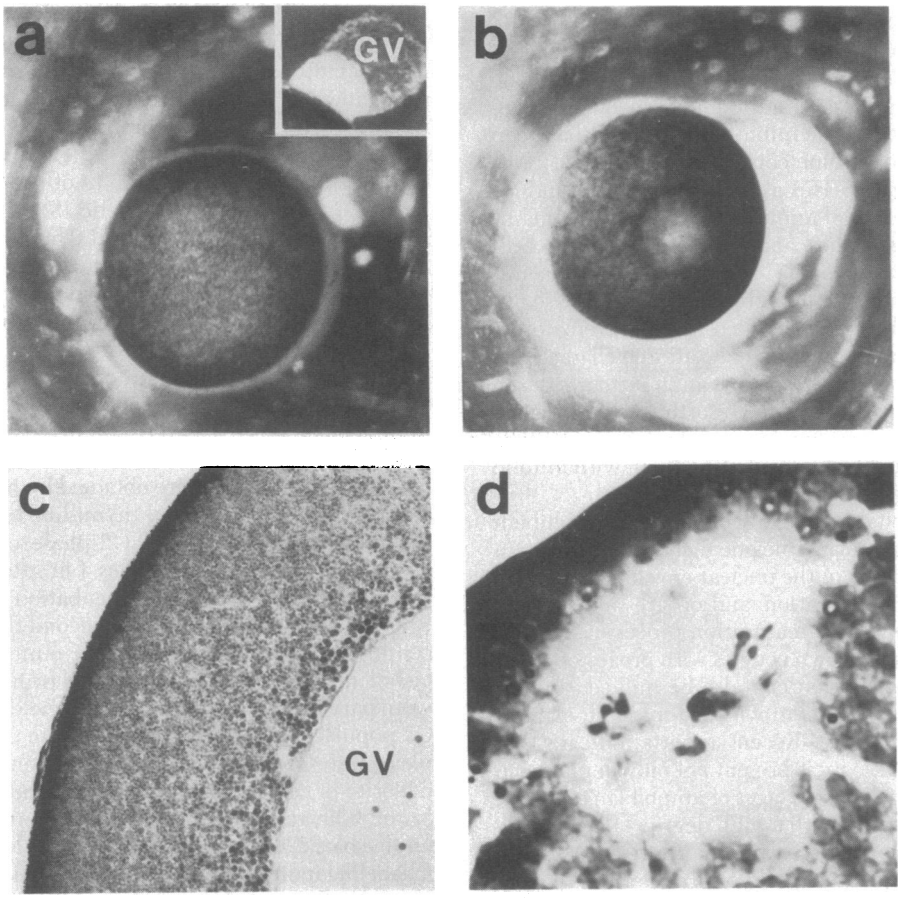

FIG. 1. GVBD and chromosome condensation in *Xenopus* oocytes injected with mitotic HeLa cell extracts. (*a*) Oocyte animal hemisphere 3 hr after injection with 65 nl of extraction medium. (×26.) (*Inset*) Clear germinal vesicle (GV) dissected from buffer-injected living oocyte. (*b*) Appearance of an oocyte animal hemisphere at 3 hr after injection with mitotic HeLa cell extract (228 ng of protein in 65 nl). Note the bright spot indicating the depigmented area caused by GVBD. (*c*) Histological section of an oocyte 4 hr after injection with S-phase HeLa cell extract (406 ng of protein in 65 nl). (Stained with Feulgen/fast green; ×236.) Note intact germinal vesicle. (*d*) Condensed chromosomes on meiotic spindle near oocyte surface 1.5 hr after injection with HeLa mitotic cell extract (309 ng of protein in 65 nl). (Stained with Feulgen/fast green; ×1750.) Appearance of chromosomes suggests an arrested second meiotic metaphase.

Table 1. Maturation-promoting activity of cytoplasmic extracts of HeLa cells from different phases of the cell cycle

Cell cycle phase	Protein injected,* ng	Oocytes injected	Oocytes showing GVBD	Induction of GVBD, %
Early G1	325	23	0	0
Late G1	293	19	0	0
S	455	22	0	0
Early G2	351	24	2	8.3
Mid-G2	325	29	2	6.9
Late G2	293	22	8	36.1
G2 arrested	358	22	0	0
Mitosis	455	15	15	100
Mitosis	228	26	26	100
Mitosis	114	16	3	18.7
Mitosis	57	16	0	0
Extracts from progesterone-stimulated oocytes	1300	15	15	100
Extraction medium	—	20	0	0

* A total volume of 65 nl of extracts was injected into each oocyte.

RESULTS

Our first objective was to find out whether the extracts from mitotic cells could induce maturation in amphibian oocytes. All the oocytes injected with mitotic extracts exhibited GVBD and chromosome condensation (Fig. 1). The positive controls, consisting of extracts from progesterone-stimulated oocytes, induced GVBD in 100% of the oocytes injected (Table 1). No maturation-promoting activity was found in the extraction medium. The activity of the mitotic extracts was dependent on the dose of the mitotic protein injected. The mitotic extracts exhibited full activity even at 50% dilution, but further dilutions resulted in a rapid loss of activity. Extracts of cells from early G1, late G1, and S phase and those arrested in G2 phase had no activity. The maturation-promoting activity that initially appeared in early G2 showed a dramatic increase in late G2 and reached a peak in mitosis (Fig. 2).

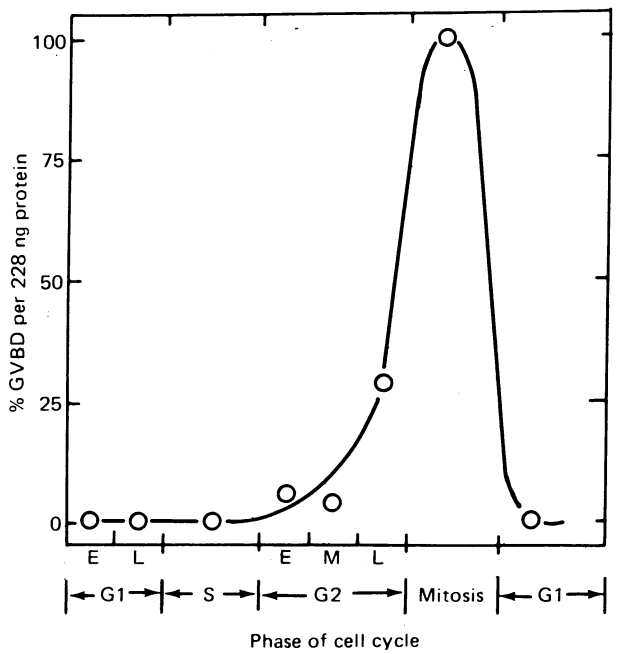

FIG. 2. Meiotic maturation-promoting activity of cell extracts during HeLa cell cycle. The data for this graph are derived from Table 1. Because 228 ng of mitotic protein induced GVBD in 100% of the cases, the percent activity for other phases of the cell cycle was normalized to that amount of protein. E, early; M, mid-; and L, late.

DISCUSSION

These data suggest the following: (*i*) Mitotic factors from mammalian cells can induce GVBD and chromosome condensation in amphibian oocytes and these factors have no species barriers. (*ii*) The factors involved in the breakdown of nuclear membrane and chromosome condensation, which are associated with three different phenomenon—viz., mitosis, meiosis, and PCC—appear to be very similar, if not identical, throughout the animal kingdom. (*iii*) The HeLa mitotic factors are as effective in inducing maturation as the extracts from progesterone-stimulated oocytes. This process takes about 1.5–2.0 hr as compared to 6–8 hr required for progesterone-induced maturation. (*iv*) Furthermore, the mitotic factors appear to be very efficient in inducing maturation of oocytes as revealed by the following calculations. Assuming that the average diameters of *Xenopus* oocytes and HeLa cells to be approximately 1400 μm and 17 μm, respectively, the estimated volume of an oocyte is about 500,000 times greater than that of a HeLa cell. To induce GVBD in 100% of the oocytes, a protein equivalent of 1000 mitotic cells has to be injected into an oocyte (Table 1). In other words, on a volume-to-volume basis the mitotic extracts were effective even at a 1:500 dilution in the oocyte. However, the nature of these factors still remains to be elucidated. Initial attempts at characterizing the maturation-promoting factors from the cytoplasms of maturing amphibian oocytes indicated that their activity was Mg^{2+}-dependent, Ca^{2+}-sensitive, and associated with a heat-labile protein (9). These studies also revealed that these factors may exist in three different molecular size aggregates and that they have no species specificity within the amphibians (9, 20). (*v*) The results of the present investigation (Fig. 2), which indicate cyclical changes in the levels of maturation-promoting activity during the HeLa cell cycle, confirm earlier observations on amphibian embryos during early cleavage (10). This knowledge is, however, further extended by the present study, which indicates the course of accumulation of mitotic factors during the cell cycle. From these results it is clear that the mitotic factors accumulate slowly in the beginning of G2 but at a progressively more rapid rate during late G2 and reach a threshold at the G2–mitotic transition when the nuclear membrane breaks down and chromatin condenses into chromosomes. In a previous study we have shown the HeLa cells arrested in G2 phase by *cis*-acid treatment were deficient in certain proteins specific to G2 to mitotic transition (14). Extracts from such G2-arrested cells fail to induce GVBD in the oocytes. This observation further supports the conclusion that mitotic factors accumulate during the G2 period. Because the factors present in the G2 cells can induce GVBD and chromosome condensation in oocyte nuclei, we suggest that the G2 cells contain active mitotic factors but at a relatively lower concentration than mitotic cells.

This study demonstrates the potential of the amphibian oocyte system for the isolation and characterization of mitotic factors from mammalian cells.

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